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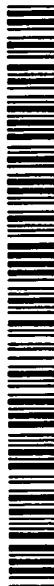


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- (54) Title: REVERSE TRANSCRIPTASE ASSAY
- (57) Abstract: A method for the detection of a reverse transcriptase (RT) activity, in particular porcine endogenous retrovirus, in a test sample is disclosed. Also provided is a kit for use in the detection of reverse transcriptase activity in a sample.
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REVERSE TRANSCRIPTASE ASSAY

The present invention relates to a method for the detection of reverse transcriptase (RT) activity, and to a method for the detection of retroviruses. The invention further relates to kits for use in the detection of RT activity and of retroviruses. The invention yet further relates to a method and kit for the detection of porcine endogenous retrovirus (PoERV).

Retroviruses are important infectious agents in humans and animals, and are responsible for a large number of diseases. These agents may be passed between hosts by, among other means, transfer of biological material, for example, organ transplants, blood transfusions and the like. A source of particular concern is xenotransplantation; that is, organs/tissues transplanted across species. There is a concern that certain retroviruses may be able to cross the species barrier, and so cause zoonoses. One such retrovirus which has been shown to be able to cross species is porcine endogenous retrovirus (PoERV); this is of particular concern for xenotransplantation, as pigs are a favoured source of suitable organs/tissues.

Reverse transcriptase (RT) activity is normally associated with the presence of retroviruses and detection of RT activity in harvest material, cell supernatants or viral vaccine preparations can be diagnostic for retroviral contamination. For many years, conventional RT testing has been performed using synthetic oligonucleotides as

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templates for RT and estimations of contaminating DNA polymerases, however the test is not sensitive enough to detect low levels of RT activity (Robertson et al., 1997; Maudru and Peden, 1998).

5 Product enhanced reverse transcriptase (PERT) assays, also termed Amp-RT or polymerase chain reaction-based reverse transcriptase (PBRT) assays (Maudru and Peden, 1997), have been reported to be up to 10^6 -fold more sensitive than conventional RT assays for detecting the
10 presence of retroviruses (Silver et al., 1993; Pyra et al., 1994; Heneine et al., 1995; Roberston et al., 1997; Arnold et al., 1998). Consequently, such tests are the first choice for detection of RT in live viral-vaccines, gene
15 therapy preparations and the screening of animals and patients for PoERV in xenotransplantation trials. The high sensitivity of product enhanced reverse transcriptase tests means that they can play a significant role in the evaluation of the retroviral status of vaccines and other biological material from the biotechnology industry where
20 safety of products is paramount. More specifically, when performed in combination with co-cultivation, $S^+ L^-$ assays, and infectivity assays, together with electron microscopy (EM) and pathogen specific PCR (polymerase chain reaction) detection assays, they can assist in providing a detailed
25 risk assessment for the presence of adventitious agents in samples (Robertson et al., 1997; Martignat et al., 1998; Kahn et al., 1998; Martin et al., 1998).

PERT assays are RT-dependent PCR assays and therefore combine the broad specificity of conventional RT assays with the high sensitivity of PCR. As with conventional RT assays, they are utilised to detect the RT activity packaged into extracellular retrovirus particles. The assays involve converting an RNA template to cDNA and then amplifying the cDNA using product-specific primers. As no exogenous RT activity is added to the reaction, cDNA will only be generated if the sample itself contains RT activity. If RT activity is not present, product will not be detected.

Detection of the PCR product may be achieved by conventional means after the end of the amplification process. However, real-time detection of levels of PCR product during amplification may be achieved using TaqMan® PCR technology with the detection of specific PCR product accomplished using for example the ABI Prism 7700 Sequence Detection System (PE Biosystems, Foster City, California). During amplification with PCR primers, a specific fluorogenic oligonucleotide probe with both a reporter and quencher dye attached anneals specifically to the amplified product between the amplimers. The detection reaction uses the 5'-nuclease activity of AmpliTaq Gold™ DNA polymerase (PE Biosystems) to cleave the reporter dye from the probe, which results in an increased fluorescence when reverse transcriptase activity is present.

A major problem with PERT assays is the potential for misinterpretation of test results, leading to the conclusion that a sample contains retroviral RT. This arises from the sensitivity of the test, coupled with the ability of DNA polymerases, telomerases and other cellular enzymes to utilize RNA templates, thereby showing RT-like activity (Lugert et al., 1996, Silver et al., 1993, Boni et al., 1996).

Arnold et al., 1998 recently developed a one-step fluorescent PERT assay that utilised the TaqMan® fluorescent probe technology with RT and an MS2 RNA template. However, there are anomalies in the data generated using this method resulting from an inappropriately high fluorescence background.

It is among the objects of the present invention to provide a high throughput fluorescent PERT (F-PERT) assay that has the ability to discriminate between positive results generated by cellular polymerases and/or other enzymes and genuine retroviral RT activity.

According to a first aspect of the present invention, there is provided a method for the detection of reverse transcriptase (RT) activity in a test sample, the method comprising the steps of:

a) contacting the test sample with an RNA template and an oligonucleotide that is complementary to a portion of the RNA template under conditions which allow the oligonucleotide and the RNA template to anneal and such that a DNA strand will be synthesised by extension from the

oligonucleotide if reverse transcriptase is present in the sample;

b) subjecting the resulting mixture to a treatment whereby any DNase present in the mixture is substantially inactivated;

c) amplifying the synthesised DNA by use of a DNA polymerase, under conditions whereby the amplified DNA may be detected by incorporation or release of a label; and

d) detecting any amplified DNA by way of the incorporation or release of the label.

As used herein, "reverse transcriptase activity" refers to biological enzymatic activity which is capable of generating a DNA strand from a template RNA strand, in the presence of suitable nucleotides, cations and buffers and the like, and under appropriate temperature and pH conditions. Typically the method is used to detect reverse transcriptase activity of a retrovirus present in the sample. The method may be used to detect any retrovirus, for example FeLV, HIV and in particular porcine endogenous retrovirus.

A "test sample" may comprise any biological tissue or body fluid (for example, cells, serum, plasma, semen, urine, saliva, sputum, cerebrospinal fluid), and may be processed in any suitable manner in order to prepare the sample for testing. For example, the test sample may be clarified by low speed centrifugation at 10,000g and filtered through a 0.45 micron filter to remove debris. After this the test sample may be tested directly after

mixing in disruption buffer. Alternatively, the sample may be centrifuged at approximately 100,000 g for 1 h to sediment any virus from a test sample to concentrate it or remove any soluble inhibitors of RT activity. The sample would still be disrupted by detergent disruption buffer. Furthermore, a test sample may be tested directly without clarification and concentration by centrifugation. An induction step may be included prior to assaying the sample in order to stimulate retrovirus production.

"Amplifying" refers to any procedure capable of increasing the number of copies of a particular nucleic acid sequence in a sample. Typically this amplification will be performed by means of the polymerase chain reaction (PCR), although it may be possible to use alternative techniques, for example, the ligation amplification reaction (LAR), or the ligase-based amplification system (LAS), and the like. In usual embodiments of the present invention, several rounds of amplification/detection steps will be performed. Moreover, the amplification step (c) and detection step (d) may occur concurrently, allowing real-time detection of the generation of amplified DNA.

The assay is as sensitive as other recently published conventional PERT (C-PERT) assays (Maudru and Peden, 1997), and at least 10^4 times more sensitive than conventional RT (C-RT) assays.

In a preferred embodiment amplification of the synthesised DNA is carried out under conditions such that during amplification a probe comprising an oligonucleotide,

possessing a reporter molecule and a suppressor molecule, anneals to a strand of the template nucleic acid and whereby nuclease activity of the DNA polymerase cleaves one of said suppressor molecule and said reporter molecule from the probe. Thereafter the non-suppressed reporter molecule may be detected.

Preferably the reporter molecule is a fluorescent molecule. Conveniently the reporter molecule is FAM (6-carboxyfluoresceine). Conveniently, the suppressor molecule is TAMRA (6-carboxy-N, N, N', N'-tetramethyl rhodamine). Preferably the complementary oligonucleotide bears FAM at its 5' end, and TAMRA at its 3' end. This system is the basis of the TaqMan® technology. Other suitable reporter and or suppressor systems may of course be used. In addition to the 3'quencher label (DABCYL), a variety of fluorophores are available for conjugation to the 5' oligo terminus or to bind directly to products of DNA amplification. These dyes include fluorescein (6-FAM), Texas Red, TET, HEX, Cy3, Cy5, TAMRA, VIC™, SYBR Green I and Sentinel™ Molecular Beacons (PE Biosystems; Stratagene).

The TaqMan® technology from ABI, as described above, represents the newest development in high throughput PCR. This system has aided the detection and quantitation of pathogen DNA and RNA (Morris et al., 1996; Kennedy et al., 1998; Higgins et al., 1998; Everett et al., 1999; Josefsson et al., 1999), and the rapid identification of retroviral reverse transcriptase activity (Arnold et al., 1998). The

incorporation of this technology into a PERT assay will be of benefit in retrovirus discovery and biosafety testing. The main advantage of the TaqMan® technology is the reduced labour and sample manipulation. As analysis is performed within a closed reaction well, post-PCR processing is minimised and the risk of contamination by carry-over PCR product is reduced significantly. The assay is also quantitative and direct quantification of RT in samples can be carried out rapidly without the need of achieving target-to-competitor ratios. Additionally, real-time PCR reactions are also less sensitive than endpoint PCR reactions to the effects of inhibitors, presumably because measurements are taken during the exponential phase where reaction components are not limiting.

The previously described assay by Arnold et al (1998) displayed a high level of background fluorescence. Without wishing to be bound by theory it is thought that this may be due to DNase present in the sample processing the probe and releasing the reporter molecule. The present assay therefore employs a DNase inactivation step. Preferably the DNase inactivation step is a high-temperature protein denaturation step. Conveniently the reaction is held at at least 90°C for at least 5 minutes; more preferably the reaction is held at at least 95°C for at least 10 minutes. Alternatively a DNase inactivator such as a proteinase may be added.

One of the major problems with RT tests and the sensitive polymerase based RT tests in particular is the generation of signals by cell-associated enzymes such as DNA polymerases and telomerases that can generate an RT-like polymerisation with RNA templates.

In preferred embodiments of the present invention, the known RNA template lacks telomerase target sequences. For example, the known RNA template may be derived from Brome Mosaic Virus (BMV) RNA, or bacteriophage MS2 RNA. Other suitable RNA templates may of course be used, for example, genomes of RNA viruses with no DNA intermediate such as influenza virus, tobacco mosaic virus or the bacteriophage of the *Leviviridae*. However, any purified RNA would provide a suitable template for RT if an appropriate control reaction were included. The sequence specificity of telomerases is such that there is no target template sequence in the BMV or MS2 fragment amplified and therefore, no potential for a signal from the RT-like activity associated with the enzyme (Blackburn 1993).

The RT reaction may be carried out at a pH above 5.5, and preferably also at a pH of below 8.5 and preferably at a pH of about 8.2. However, the reaction may be carried out at a pH of 3 to 10 but is not efficient at the extremes of this pH range. Several laboratories have employed methods that suppress the RT-activity associated with cellular DNA polymerases (Lugert et al., 1996; Chang et al., 1997). Chang et al., 1997 have reported that authentic RT activity has a broader pH range than cellular

polymerase RT-like activity. They have reported that lowering the pH of the RT reaction to pH 5.5 suppresses non-retroviral RT activity. However, they demonstrated that RT or RT-like activity was not detected in CHO and BHK-21 cell lysates at the lower pH of pH 5.5. This is of concern as endogenous retroviral particles and RT activity have been detected in association with CHO (Anderson et al., 1991; Dinowitz et al., 1992; Adamson, 1998) and BHK-21 (DeHaven et al., 1998) cells (Table 6). Therefore, it is likely that lowering the pH of the RT reaction has detrimental affects on the detection of authentic RT activity of some retroviral types and may allow the misinterpretation of data and the reporting of false RT-negative test samples.

Under certain conditions, the known RNA template is at a concentration of less than 6 ng/ μ l in the reaction mix. In a preferred protocol, the RNA concentration may be less than 1.5 ng/ μ l in the reaction mix. Cell lysates provide a source of cell-associated polymerases and the signal generated from such samples was not observed on reduction of the BMV RNA template level from 60 ng to 15 ng and below. However, the detection of RT activity was unaffected. This observation may reflect a reduced affinity for RNA templates of DNA polymerases. In addition, the generation of RT-like activity by DNA polymerases was less reproducible than the genuine retroviral RT activity observed.

Preferably the method step a) includes a suppressor of RT activity of DNA polymerases. Typically the suppressor may be activated DNA. For example the suppressor may be activated calf thymus DNA (aCT DNA). The suppressor may be included in the reaction mix in excess of the known RNA template; in the case of aCT DNA, this is for example a ratio of at least 1:26 template: aCT; or more preferably at least 1:104, and most preferably at least 1:416. In a typical protocol, the concentration of aCT DNA in the RT reaction mix is at least 150 ng/ μ l.

Activated calf thymus DNA (aCT) has previously been observed to essentially suppress the positive signals produced by DNA polymerases with no reduction in RT activity (Lugert et al., 1996). In a preferred embodiment there is disclosed the use of aCT in a similar manner to further reduce interference from cellular polymerases and to enable a test operator to distinguish between retroviral RT activity and high levels of DNA polymerases activity in a sample. The incorporation of aCT DNA into the RT reaction of the presently described assay at increased ratios as compared to the RNA template suppresses the RT-like activity from purified cellular polymerases.

According to a further aspect of the present invention, there is provided a kit for use in the detection of reverse transcriptase (RT) activity in a sample, the kit including a combination of the necessary reaction reagents, oligonucleotides, RNA templates activated DNA and the like for use in the methods outlined above.

According to a still further aspect of the present invention, there is provided a kit as described above for use in the detection of porcine endogenous retrovirus (PoERV) in a test sample. Such a kit may include a porcine endogenous retrovirus specific RNA template. Details of suitable PoERV specific RNA templates may be found in co-pending patent applications WO97/40167, WO97/21836 and GB9919604.0.

The PoERV RNA template may be synthesised *in vitro* and may for example correspond to positions encompassing the positions 805 to 902 of the PoERV genome (see Figure 3 of WO97/40167).

The PoERV-gag-specific oligonucleotides used would be derived from sequences which correspond to approximate positions 805-823 (Forward), to position 828 to 854 (Probe) and position 902-877 (Reverse) on the PoERV genome (see Figure 3 of WO97/40167).

The oligonucleotide primers used for this amplification would be as follows:

Forward primer 5'-CCGGCTCTCATCCTGATCA-3'

Reverse primer 5'-TCTTGTTTATTTAGCCATGGTTTAA-3'

The fluorescent 5'-FAM (6-carboxy fluoresceine) and 3'-TAMRA (6-carboxy-N, N, N',N'-tetramethylrhodamine) quencher labelled probe would be as follows:

Probe 5'-CCCTATATCCTTACGTGGCAAGATTTG-3'.

Alternatively it may be possible to use the natural primer (glycine 2 tRNA) with the primer binding site of the PoERV genome, the PBS (primer binding site) in the U5 region of the LTR (long terminal repeat).

5 These and other aspects of the present invention will now be described by way of example only, with reference to the accompanying figures, in which:

10 Figure 1 shows (a) the relationship between C_T (threshold cycle) value and number of MLV virions in experiments carried out in accordance with the above method; and (b) the relationship between cycle number and ΔR_n value for various numbers of MLV virions as shown on the graph of part (a);

15 Figure 2 shows a list of cell lines and virus stocks used in the following examples: Abbreviations: ATCC (American Type Culture Collection), ECACC (European Collection of Animal Cell Cultures), DVPGU (Department of Veterinary Pathology, University of Glasgow), ABInc (Advanced Biotechnologies Inc.).

20 Figure 3 shows detection limits using the presently described F-PERT assay for both retroviral particles and purified RT enzyme; (A) retroviral particles (B) purified RT enzyme. A test is considered positive when a given sample with a minimum of four replicated wells resulted in
25 a C_T value equivalent to or below 32. Letters in superscript indicate identical retroviral stocks of each viral type. ^aPoERV, ^bMLV, ^cSMRV.

13(12)

Figure 4 shows a comparison of the sensitivity of retroviral RT activities for both the present F-PERT assay and conventional RT assays; The detection limit is expressed as the highest dilution detected using each assay.

Figure 5 shows the effect of divalent cation concentration on RT activity; The mean C_t values obtained from at least three replicates and the 95% confidence limits are shown in brackets. Abbreviations; CL; 95% confidence limit.

Figure 6 shows the suppression of RT-like DNA polymerase activity by aCT DNA; (A) Retroviral negative cell lysate MRC-5 and affect of BMV RNA template concentration and increasing activated calf thymus DNA (aCT) ratios on RT-like activity (B) Amplification results obtained from 1-5 unit (U) of enzyme which gave the highest activity in F-PERT reactions without aCT DNA and suppression of that activity by aCT DNA (BMV: aCT ratio 1:104). Abbreviations; *Taq*; (5U native *Taq* DNA polymerase). α ; (1U calf thymus DNA polymerase α). β ; (1U human DNA polymerase β). δ ; 0.1U DNA polymerase δ and 75ng of δ -PCNA cofactor. *TdT*; 1U terminal deoxynucleotide transferase. *SMRV*; 10^3 *Squirrel monkey retrovirus*; and

Figure 7 shows the RT activity of various cell line supernatants, as tested by the present method. Cell culture supernatants were tested with and without activated calf thymus DNA as indicated. IDU-induced cultures are

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identified by superscript ^a. Abbreviations; (+) positive
and (-) negative for RT-like activity by $C_T \leq 32$ or > 32
respectively. Supernatants spiked with 10^6 rVLPs of a RT
5 positive stock are shown as superscript ^b. Those labelled
as superscript ^c were spiked with 10^5 rVLPs. All other
supernatants were spiked with 10^3 rVLPs. NT: not tested.

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The specificity of the conventional RT and F-PERT methods were tested using a wide range of retroviral RT with different cation preferences and compared the sensitivities of each assay. Depending on the retrovirus, present F-PERT assay was between three and six \log_{10} more sensitive than conventional RT assays and can detect RT from a wide range of retroviruses.

Assay sensitivity was measured using dilutions of preparations of MLV (murine leukaemia virus), SMRV (squirrel monkey retrovirus) and PoERV (porcine endogenous retrovirus) containing a known number of retroviral particles. In addition dilutions of purified RT enzyme from Avian myeloblastosis virus (AMV) and Moloney-MLV were tested. The present F-PERT assay was able to detect 10^2 MLV and 10^1 SMRV particles. Analysis of PoERV rVLPs (retrovirus - like particles) at similar dilutions indicates that the present F-PERT assay was able to detect 10^2 rVLPs. Purified RT enzyme was detected at 10^1 molecules for AMV and MLV using F-PERT. These levels of detection of purified enzymes are consistent with the data derived from virion particles, assuming a range of approximately 40-100 molecules per particle (Panet et al., 1975; Krakower et al., 1997).

The Gammaretrovirus PoERV has received considerable attention owing to the possible intimate contact with the human host during xenotransplantation (Patience et al., 1997; Patience et al., 1998; Takeuchi et al., 1998). The present F-PERT assay was optimised to include the detection

of the PoERV reverse transcriptase enzyme.

The present F-PERT assay can allow the discrimination of RT generated by infectious extracellular particles, and any RT activity that is associated with the endogenous transcription of the provirus *pol* gene inside the infected cell nucleus without production of viral particles.

The present F-PERT assay was able to detect endogenous retroviral activity from BHK-21, CHO, C127I, *Mus dunni* and Sf-9 cell supernatants. Endogenous retroviral particles with reverse transcriptase activity have been detected in numerous species including invertebrates (Gabriel and Boeke, 1993; Hajek and Friesen, 1998), Chinese and Syrian hamsters (Anderson et al., 1991; Dinowitz et al., 1992; Adamson, 1998; DeHaven et al., 1998), feline (Baumann et al., 1998), murine (Wolgamot et al., 1998) and *Homo sapiens* (Berkhout et al., 1999).

METHODS

Cell lines and Culture Conditions

Cell lines used in this study are shown in Figure 2. Mammalian cell lines were cultured at 37°C in foetal calf serum supplemented Dulbecco's modified eagles medium (DMEM) or RPMI 1640 medium (Life Technologies Ltd., UK). Insect cells were cultured in SF900-II medium (Life Technologies Ltd., UK). Supernatants were harvested from the growing cultures when healthy, normally 2-5 days at 37°C post subculture and stored at or below a temperature of -70°C.

For induction of retrovirus from cell lines, duplicate cell cultures were initiated in tissue culture medium and after 24 hours 5-iodo-2-deoxyuridine (IDU; Sigma-Aldrich Co. Ltd) to a final concentration of 20 μ g/ml was added to one of the cultures. The cultures were incubated overnight then fed with appropriate medium if necessary. Three days later, supernatant was harvested from the growing cultures and assayed for reverse transcriptase activity.

Preparation of Virus Stocks and Determination of Retrovirus Particle Concentration

The retroviruses used in this study are indicated in Figure 2. An aliquot of each retrovirus was inoculated onto the appropriate detector cell lines, in the presence of 10 μ g/ml polybrene (Sigma-Aldrich Co. Ltd), incubated at 37°C for approximately 90 minutes and fed with appropriate culture medium. The inoculated cultures were maintained for 2-5 passages and supernatants harvested 2-5 days post subculture. Supernatants were centrifuged to remove cellular debris, filtered through a 0.45 μ m filter unit, and stored in single use aliquots at or below -70°C.

Electron Microscopy

Negative stain electron microscopy (NSEM) (Doane, 1980) was used to quantitate the virion stocks. Viral particles were pelleted by ultracentrifugation at 100,000 g for 60 min, followed by resuspension in DMEM (Life Technologies Ltd.). A selected volume of sample was mixed with an equal volume of latex spheres of known titre (Agar

Scientific) and diluted if necessary. The sample was then applied to a pioloform-coated EM mesh grid and allowed to air dry. The grid was fixed with 2.5% (v/v) glutaraldehyde, stained with 5% uranyl acetate and allowed to air dry completely. Samples were visualised using a Philips EM-400 transmission electron microscope. Retrovirus particles were recognised by their size (80-120nm) and structural features (concentric circles containing a dark inner core). Latex spheres and retrovirus-like particles (rVLPs) were counted in parallel until 1000 latex beads were enumerated and the total particle count of the original volume calculated and retrovirus concentrations were recorded.

Test Sample Preparation

Appropriate volumes and dilutions of virus stocks and cell line supernatants were clarified by centrifugation at approximately 11,000 g for 10 minutes, then the sample passed through a 0.45 μ m sterile filter to remove remaining debris. Retroviruses were concentrated from supernatants by ultracentrifugation at 100,000 g for 60 minutes. To assess the level of inhibition of the F-PERT assays by test samples, the samples were spiked with a known number of retroviral-like particles prior to ultracentrifugation. Each pellet was resuspended in disruption buffer (40 mM Tris-HCl pH 8.1, 50 mM KCl, 20 mM dithiothreitol, 0.2% (v/v) NP-40) (Sigma-Aldrich Co. Ltd) and appropriate dilutions prepared. Typically 5-25 μ l volumes of samples

were used immediately in RT reactions.

Cell lysates were prepared by suspending washed cell pellets in 200 μ l of disruption buffer containing a Complete™ EDTA-free protease inhibitor cocktail (Boehringer Mannheim, Cat. No. 1-836-170), and incubated on ice for 20 min. Total cellular protein concentration was measured using a protein assay kit (Sigma Diagnostics Cat. No. P5656). RT reactions were carried out with 25 μ g, 10 μ g, 1 μ g and 100 ng of total cell lysate protein.

Reverse Transcriptase Detection Assays

Oligonucleotides, Reagents and Purified Enzymes

Synthetic oligonucleotide primers and probes used in the present F-PERT assay for Brome mosaic virus RNA and MS2 RNA were obtained from PE Biosystems Ltd. UK:

Brome mosaic virus:

Forward primer (5')-TCTTGAGTTAGACCACAACGTTTCCT-(3')

Reverse primer (5')-TGCGCTTGTCTCTGTGTGAGA-(3')

The fluorescent 5'-FAM (6-carboxy fluoresceine) and 3'-TAMRA (6-carboxy-N, N, N',N'-tetramethyl rhodamine) quencher labeled probe was as follows:

(5')FAM-TCTGCTCGAGGAGAGCCCTGTTCC-TAMRA(3').

For bacteriophage MS2 RNA the primers were:

Forward primer (5')-GGCTGCTCGCGGATACC-(3')

Reverse primer (5')-AACTTGCGTTCTCGAGCGAT-(3')

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The fluorescent 5'-FAM (6-carboxy fluoresceine) and 3'-TAMRA (6-carboxy-N, N, N',N'-tetramethyl rhodamine) quencher labeled probe was:

(5') FAM-ACCTCGGGTTTCCGTCTTGCTCG-TAMRA(3').

DNA polymerases

The following purified enzymes were tested for their ability to produce RT-like DNA polymerase activity in the F-PERT test: calf-thymus DNA polymerase α (Cambio, UK), human DNA polymerase β (Chimerx, Millwaukee, USA), calf liver polymerase γ (Raimond Lugert, Paul-Ehrlich-Institut, Langen, Germany), DNA polymerase δ and PCNA (Dmitry Mozzherin, State University of New York at Stony Brooke, USA), native *Taq* DNA polymerase and terminal deoxynucleotide transferase (Life Technologies Ltd., UK). Aliquots of purified enzymes containing 1 unit to 1 milliunits in ten fold series were tested in the absence of activated calf thymus DNA (aCT) for their ability to produce RT-like polymerase activity in the F-PERT assay. The amount of enzyme showing the highest RT-like activity was then tested in the presence and absence of aCT DNA.

Purified reverse transcriptase enzymes used in the study were Avian myeloblastosis virus (AMV: molecular weight 160kD, specific activity 98210 units/mg, Boehringer Mannheim cat. #. 1495-062) and recombinant Moloney-Murine leukaemia virus (M-MLV: molecular weight 71kD, specific activity 4×10^4 units/mg, Boehringer Mannheim cat. #. 1062-

603).

Procedure and terminology used in the present F- PERT assay

Terminology

5 The ABI Prism™ 7700 sequence Detection System (PE Biosystems) uses the 5'-nuclease activity of AmpliTaq Gold™ DNA polymerase to cleave a probe consisting of an oligonucleotide with a reporter dye at the 5'-end and a quencher dye at the 3' -end. The quencher is only cleaved from the probe when the probe is hybridised with the target DNA and the increase in fluorescence of the probe can be measured. The system detects and calculates a reporter dye value (R_n) for each sample during each cycle of amplification. The value of R_n , or the normalised reporter signal, represents the fluorescence of the reporter dye divided by the passive reference dye. During PCR, R_n increases as amplicon copy number increases until the reaction approaches a plateau. In this application data was analysed using real-time PCR and the fluorescent signal was read continuously throughout cycling. The value ΔR_n represents the normalised reporter signal R_n minus the baseline signal established in the first few cycles of PCR. Like R_n , ΔR_n increases during PCR as amplicon copy number increases until the reaction reaches a plateau. The C_t , or threshold cycle value is calculated in real time and represents the PCR cycle at which an increase in reporter fluorescence above the baseline signal can first be detected. The greater the amount of PCR template the lower

the threshold cycle (PE Biosystems TaqMan® Gold RT-PCR kit, 1997).

Reagents used in PCR amplification were either the TaqMan® Universal PCR Master-mix (PE Biosystems #P/N 4304437) or the TaqMan® Gold RT-PCR kit (PE Biosystems # P/N808-0233). Amplification was quantified in real time by measuring the accumulation of fluorescent signal obtained from the 5'-nuclease activity of AmpliTaq Gold™ and the release of the reporter dye from the TaqMan® probe during the standard amplification cycles. RT activity is expressed using 95% confidence limits of threshold cycle (C_T) values, where C_T is the cycle at which a statistically significant increase in fluorescence is first detected.

A minimum of three replicates were used for each reaction in a 96 well plate format (MicroAmp® Optical 96-Well Reaction Plate # N801-0560).

Procedure for the F-PERT assay

RT activity was measured from cell line lysates and culture supernatants and purified reverse transcriptase enzymes using the exogenous RNA template from the Brome mosaic Virus (BMV) (Promega, UK) or bacteriophage MS2 (Boehringer Mannheim). For reverse transcriptase reactions, the TaqMan® Gold RT-PCR kit (PE Biosystems # P/N808-0233) was prepared at 2X concentration. An equal volume of test samples prepared in disruption buffer were added to the 2X RT reaction mix resulting in a final concentration of 50 mM KCl, 10 mM Tris-HCl pH8.3, 5.5 mM

MgCl₂, 500 μM dATP, dCTP, dGTP and dTTP, 0.2 μM reverse primer, 0.1% (v/v) NP-40 (Sigma-Aldrich Co. Ltd), 10mM dithiothreitol (Sigma-Aldrich Co. Ltd), 0.4U/μl placental RNAGuard (Amersham Pharmacia Biotech; #27-0815-01). RT reactions contained BMV RNA (Promega) to aCT (Sigma-Aldrich Co. Ltd) ratios of 1:26, 1:52, 1:104, 1:208, 1:416 and 1:832 when appropriate (157 ng/μl final concentration of aCT DNA in the RT reaction). Separate air spaces were used for the reagent preparation, test sample and spiked test sample processing, and amplification of cDNA products (Ou et al., 1991). A minimum of three replicates were used for each reaction in a 96 well plate format. Sentinel controls for airborne contamination consisted of open wells containing the reagents described above and an equal volume of disruption buffer (Saksena et al., 1991). The mixes were incubated in an ABI 7700 sequence detection system at 48°C for 30 min for the RT step. Then the PCR reaction mix was added to each tube and the standard amplification was done: 10 min at 95°C to denature nucleases followed by forty amplification cycles of 15 sec at 95°C and 1 min at 60°C (PE Biosystems 1997).

The RT activity was quantified in real time by measuring the accumulation of fluorescent signal obtained from the 5'-nuclease activity of AmpliTaq Gold™ and the release of the reporter dye from the TaqMan® probe during amplification. RT activity is expressed using 95% confidence limits of threshold cycle (C_T) values, where C_T is the cycle at which a statistically significant increase

in fluorescence is first detected.

Procedure for Conventional-RT Assay

The conventional RT assay measured the activities of reverse transcriptase with poly(rA)-oligo(dT) and the activities of contaminating DNA polymerase with poly(dA)-oligo(dT) (Temin and Baltimore, 1972; Kacian and Speilgelmen, 1974; Kornberg, 1980). Retrovirus samples prepared as described above were added to equal volumes of reaction mixes containing 1 mM MnCl_2 (Sigma-Aldrich Co. Ltd), 0.025 A_{260} units of poly(dA) or poly(rA) (Amersham Pharmacia Biotech), 80 mM Tris-HCl pH 8.0 (Sigma-Aldrich Co. Ltd), 100 mM KCl (Sigma-Aldrich Co. Ltd), 0.1 mCi ^3H -TTP (Amersham Pharmacia Biotech); or 10 mM MgCl_2 (Sigma-Aldrich Co. Ltd) 0.05 A_{260} units of poly(dA) or poly(rA) (Amersham Pharmacia Biotech), 80mM Tris-HCl pH 8.0 (Sigma-Aldrich Co. Ltd), 100mM KCl (Sigma-Aldrich Co. Ltd), 0.1 mCi ^3H -TTP (Amersham Pharmacia Biotech). After incubation at 37°C for 1 hour, the trichloroacetic acid (TCA) (Sigma-Aldrich Co. Ltd) precipitated material was filtered, washed with 10% TCA, 1% sodium pyrophosphate, followed by a wash with 5% TCA, 1% sodium pyrophosphate. Incorporation of ^3H -TTP was measured by calculating the disintegrations per minute of the reaction on a Beckman Scintillation counter.

RESULTS

Assay Sensitivity, Repeatability and Linearity

During validation of the present F-PERT assay, statistical analysis of multiple observations at different time points carried out at the limits of detection and on negative control reactions containing no RT indicated that C_T values equivalent to or below 32 should be considered positive or suspect for reverse transcriptase activity. C_T values greater than 32 are in the lower boundary of the limit of detection, and therefore considered RT negative (Figures 1, 3a and 3b). To demonstrate that the F-PERT assay has the potential to be utilised as a quantitative assay, linearity was tested using 10-fold dilutions of MLV rVLPs. A standard curve was generated which indicates a linear relationship between C_T value and the number of MLV virions (correlation co-efficient of 0.990; Figure 1). The standard curve intercepted the y-axis at $C_T=37.82$ and had a slope of -4.0.

Assay sensitivity was measured using dilutions of retroviral preparations containing a known number of retroviral particles. In addition, dilutions of purified RT enzyme from AMV and M-MLV were tested in the range of 10^3 - 10^1 molecules per reaction. F-PERT reactions were done on dilutions of identical stocks of MLV, PoERV and SMRV with retrovirus particle counts of 2.9×10^6 , 2.0×10^8 and 2.8×10^7 per ml respectively. The mean coefficient of variation for the particle counting assay by transmission EM was 16.7%. The present F-PERT assay was able to detect

10² MLV and 10¹ SMRV. Analysis of PoERV rVLPs at similar a dilution series indicates that the present F-PERT assay was able to detect 10² rVLPs (Figure 3a). Purified RT enzyme was detected at 10¹ molecules for AMV and MLV (Figure 3b).

5 Additional independent retrovirus stocks were tested, which allowed an assessment of assay variation for F-PERT. The basis of assay repeatability or intra-assay variation was demonstrated by measuring the RT activity in multiple reactions of a series of dilutions of two different batches
10 of MLV and SMRV under the same operating conditions over a short interval of time. The coefficient of variation (CV) for the two different batches of MLV at 10⁴ particles was 4.3% and 3.7%. The CVs from the two different batches of SMRV at 10³ particles were 1.7% and 2.2%. With at least
15 eight replicated reactions of 10⁴-10¹ rVLPs from each MLV and SMRV batch, we were able to demonstrate that multiple samples containing 10³ rVLPs of SMRV and MLV gave similar C_t values, as shown by the narrow window of the 95% confidence limit data (Figure 3a).

20 The intermediate precision of the assay was measured by determining the limit of detection of one identical stock of MLV, SMRV and PoERV using two independent operators on separate occasions. Operator # 1 was able to detect 10², 10³ and 10¹ rVLPs of MLV, PoERV and SMRV
25 respectively. Operator # 2 was able to detect with 95% confidence positive reactions containing 10², 10⁴ and 10² rVLPs of MLV, PoERV and SMRV respectively.

Assay Specificity

Reverse transcriptase activity, when assayed in conventional RT assays using incorporation of radiolabelled nucleotides is dependent on the presence of divalent cations, either Mn^{2+} or Mg^{2+} , in the reaction mix (Temin and Baltimore, 1972; Klement and Nicolson, 1977). Firstly, using the conventional RT assay we determined the cation preference and specificity by measuring the activity in the presence of both cations. We then determined the assay sensitivity of these retroviral types using F-PERT and compared these to those obtained from conventional RT and PERT. The results demonstrated that the RT activity is greatly reduced if the alternative cation is used during the conventional RT assay. The F-PERT assays were done in the presence of 11 mM and 5 mM Mg^{2+} respectively and detected different retroviral RT with increased sensitivity regardless of cation preference (Figure 4). Several studies have compared detection of different viruses in conventional RT and PERT assays, and demonstrated the high sensitivity and specificity of PERT (Pyra et al., 1994; Heneine et al., 1995; Yamamoto et al., 1996;).

Detection of PoERV by conventional RT and C-PERT assays was relatively insensitive. Therefore the optimal conditions for the detection of the Mn^{2+} dependent retrovirus PoERV were examined by assessing the effect of varying Mn^{2+} and Mg^{2+} concentrations in the RT reaction (Phan-Thanh et al, 1992). The F-PERT assay was performed on 10^6 rVLPS of PoERV and MLV in the presence of 0.15-2 mM Mn^{2+} .

The PoERV RT was also tested at varying concentrations of Mg^{2+} cation. The RT activity of MLV was shown to be similar at all Mn^{2+} and Mg^{2+} divalent cation concentrations tested. In the presence of Mn^{2+} the optimal cation concentration for PoERV was 2 mM. However, there was no significant difference in RT activity when either Mn^{2+} at 2 mM, or Mg^{2+} at 5 mM was used in the PoERV RT reaction (Figure 5).

Suppression of positive signals generated by non-retroviral Reverse Transcriptase activity.

Cellular enzymatic activity other than retroviral RT was tested for the ability to produce false positive signals in the F-PERT assay. Cell lysates from retrovirus negative cell lines under certain assay conditions can generate these false signals in C-PERT assays (Lugert et al., 1996; Chang et al., 1997). To address this issue in the F-PERT assay the lowest amount of BMV RNA template that allowed the detection of 10^3 rVLPs with no inhibition of RT in the presence of increasing ratios of act DNA was determined. It was possible to detect 10^3 SMRV with as little as 1 ng of BMV RNA per reaction in the presence of activated calf thymus DNA (BMV: act DNA ratio of 1:832), with no inhibition of RT activity and no significant differences in activity between different BMV template concentrations. No deviations from assay linearity were detected at different BMV to act ratios.

Secondly, using MRC-5 cells with no detectable supernatant retroviral activity and by using C_t values to calculate RT equivalents the highest amount of MRC-5 total cellular lysate protein that gave the strongest false positive signal with no inhibition of RT activity (1 μ g of lysate protein) was determined. The extract was then analysed at this level along with an equivalent activity of retroviral RT from 10^3 SMRV rVLPs. By decreasing the amount of BMV RNA template in two-fold series, conditions were established where signals from the cellular polymerases were suppressed, leaving the detection of RT activity unaffected. Elimination of any positive signals produced by the cellular polymerases from the retrovirus negative cell line MRC-5 was achieved with 15 ng of BMV per reaction. Positive activity was not observed in the absence of aCT DNA at 15ng of BMV RNA per reaction and below, indicating that under certain testing conditions RT-like DNA polymerase activity may be dependent on a higher concentration of BMV template in the RT reaction. Moreover, the significant RT activity produced by 10^3 SMRV was detectable with as little as 1ng of BMV RNA and with a BMV to aCT DNA ratio as high as 1:836 (Figure 6a). Similar results were seen with MS2 RNA template.

At concentrations of lysate protein above 1 ug, the RT activity from samples spiked with 10^3 SMRV rVLPs and the RT-like DNA polymerase activity from these cellular lysates was not detected due to the presence of inhibitors.

The presence of aCT suppresses the RT activity of DNA polymerase α and γ in PERT reactions. F-PERT was done with a number of different DNA polymerising enzymes. A ten fold dilution series of purified DNA polymerising enzymes were tested in the absence of calf thymus DNA for their ability to produce RT-like polymerase activity in the F-PERT assay. The highest amount of enzyme showing the greatest RT-like activity was then tested in the presence and absence of aCT DNA. Using a BMV RNA to aCT DNA ratio of 1:102 all RT-like activity from purified DNA polymerase enzymes was suppressed in the presence of aCT DNA (Figure 6b).

EXAMPLE 1

Detection of a range of retroviral RT activity

Depending on the retrovirus, the data illustrate that F-PERT is between three and six \log_{10} more sensitive than conventional RT assays and can detect a wide range of retroviruses (Figure 4), including representatives of the Deltaretrovirus family (formally Type-D retroviruses) (Squirrel monkey retrovirus; SMRV, Simian retrovirus; SRV-1) and Gammaretrovirus family (formally Type-C retroviruses) (Murine leukaemia virus; MLV, Porcine endogenous retrovirus; PoERV), Lentivirus (Simian immunodeficiency virus; SIV), and Spumavirus (Simian foamy virus; SFV).

EXAMPLE 2**Detection of RT Activity in Cell Culture Supernatants**

A range of retrovirus infected and non-infected cell culture supernatants were tested using the present F-PERT assay (Figure 7). RT activity was not detected in non-induced Vero, Raji, MRC-5, BT, *Mus dunni* and C127I cell supernatants. Treatment of *Mus dunni* and C127I cells with IDU resulted in induction of RT activity, indicating the presence of retroviral particles. RT activity was detected in non-induced Sf-9, K-Balb, NSO, SP2/0-Ag14 and BHK-21 cell supernatants. Induction of the endogenous retrovirus from K-Balbc cells resulted in a significant increase in RT activity in cellular supernatant. As shown in Figure 7, the F-PERT assay inhibits the RT-like activity produced by cellular DNA polymerases of Vero and *Mus dunni* cells, further supporting the suppression of false positive signals by aCT DNA. RT activity is not detected in cellular lysates and supernatants from MRC-5, BT, VERO, or Raji cells, whereas RT activity was detected in C127I, *Mus dunni*, K-Balb, BHK-21, SP2/0-Ag14 and NSO cell supernatants. RT activity was also detected in the *Spodoptera* cell line Sf9.

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CLAIMS

1. A method for the detection of reverse transcriptase (RT) activity in a test sample, said method comprising the steps of:

- 5 a) contacting the test sample with an RNA template and an oligonucleotide that is complementary to portion of the RNA template under conditions which allow the oligonucleotide and the RNA template to anneal and such that a DNA strand will be synthesised by extension from the oligonucleotide
10 if reverse transcriptase is present in the sample;
b) subjecting the resulting mixture to a treatment whereby any DNase present in the mixture is substantially inactivated;
c) amplifying the synthesised DNA by use of a DNA
15 polymerase, under conditions whereby the amplified DNA may be detected by incorporation or release of a label; and
d) detecting any amplified DNA by way of the incorporation or release of the label.

20 2. The method according to claim 1 wherein the reverse transcriptase activity detected is derived from a retrovirus.

25 3. The method according to claim 2 wherein the retrovirus detected is selected from the group consisting of FeLV, HIV and porcine endogenous retrovirus.

4. A method according to claim 3 wherein the retrovirus detected is porcine endogenous retrovirus.

5. A method according to any preceding claim wherein the test sample comprises biological tissue or body fluid.

6. A method according to claim 5 wherein the test sample is selected from the group consisting of cells, serum, plasma, semen, urine, saliva, sputum and cerebrospinal fluid.

7. A method according to claims 5 or 6 wherein said test sample is processed in a suitable manner in order to prepare the sample for testing.

8. A method according to any preceding claim wherein an induction step is included prior to assaying the sample in order to stimulate retrovirus production.

9. A method according to any preceding claim wherein amplification of the synthesised DNA is carried out under conditions such that during amplification, a probe comprising an oligonucleotide, possessing a reporter molecule and a suppressor molecule, anneals to a strand of the template nucleic acid and whereby nuclease activity of the DNA polymerase cleaves one of said suppressor molecule and said reporter molecule from the probe and the non-suppressed reporter molecule is detected.

10. A method according to claim 9 wherein the reporter molecule is a fluorescent molecule.

11. A method according to claims 9 or 10 wherein the reporter molecule is FAM (6-carboxyfluoresceine).

12. A method according to claims 9 or 10 wherein the suppressor molecule is TAMRA (6-carboxy-N, N, N', N'-tetramethyl rhodamine).

13. A method according to claim 11 or 12 wherein the complementary oligonucleotide bears FAM at its 5' end and TAMRA at its 3' end.

14. A method according to claim 1 wherein the DNase inactivation step is a high-temperature protein denaturation step.

15. A method according to claim 14 wherein the DNase inactivation step comprises holding the reaction mixture at a temperature of at least 90°C for a time of at least 5 minutes.

16. A method according to claim 15 wherein the DNase inactivation step comprises holding the reaction mixture at a temperature of at least 95°C for a time of at least 10 minutes.

17. A method according to claim 1 wherein the DNase inactivator is a proteinase.

18. A method according to claim 1 wherein the known RNA template lacks telomerase target sequences.

19. A method according to claim 18 wherein the known RNA template is selected from the group consisting of Brome Mosaic Virus (BMV) RNA, bacteriophage MS2 RNA and genomes of RNA viruses with no DNA intermediate.

20. A method according to claim 1 wherein the reverse transcriptase reaction is carried out at a pH of above 5.5 and below 8.5.

21. A method according to claim 20 wherein the pH is about 8.2.

22. A method according to claim 1 wherein the known RNA template is at a concentration of less than 6 ng/ μ l in the reaction mix.

23. A method according to claim 22 wherein the known RNA template is at a concentration of less than 1.5 ng/ μ l in the reaction mix.

24. A method according to claim 1 wherein said method

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includes a suppressor of reverse transcriptase activity of DNA polymerases.

25. A method according to claim 24 wherein the suppressor of reverse transcriptase activity of DNA polymerases is activated DNA.

26. A method according to claim 25 wherein the suppressor is activated calf thymus DNA (aCT DNA).

27. A method according to claim 24, 25 or 26 wherein the suppressor is included in the reaction mix in excess of a known RNA template.

28. A method according to claim 27 wherein the ratio of activated calf thymus DNA to known RNA template is at least 1:26.

29. A method according to claim 1 wherein the concentration of activated calf thymus DNA in a reverse transcriptase reaction mix is at least 150 ng/ μ l.

30. A method according to claim 1 wherein activated calf thymus DNA is used to further reduce interference from cellular polymerases and to enable a test operated to distinguish between retroviral reverse transcriptase activity and high levels of DNA polymerase activity in a sample.

31. A kit for use in the detection of reverse transcriptase activity in a sample, said kit including a combination of the necessary reaction reagents, oligonucleotides, RNA templates, activated DNA and the like for use in the methods outlined above.

32. A kit according to claim 31 wherein said kit is used in the detection of porcine endogenous retrovirus (PoERV) in a test sample.

33. A kit according to claim 32 wherein said kit includes a porcine endogenous retrovirus specific RNA template.

34. A kit according to claim 33 wherein the PoERV RNA template is synthesised in vitro and corresponds to a sequence encompassing the positions 805 to 902 of the PoERV genome.

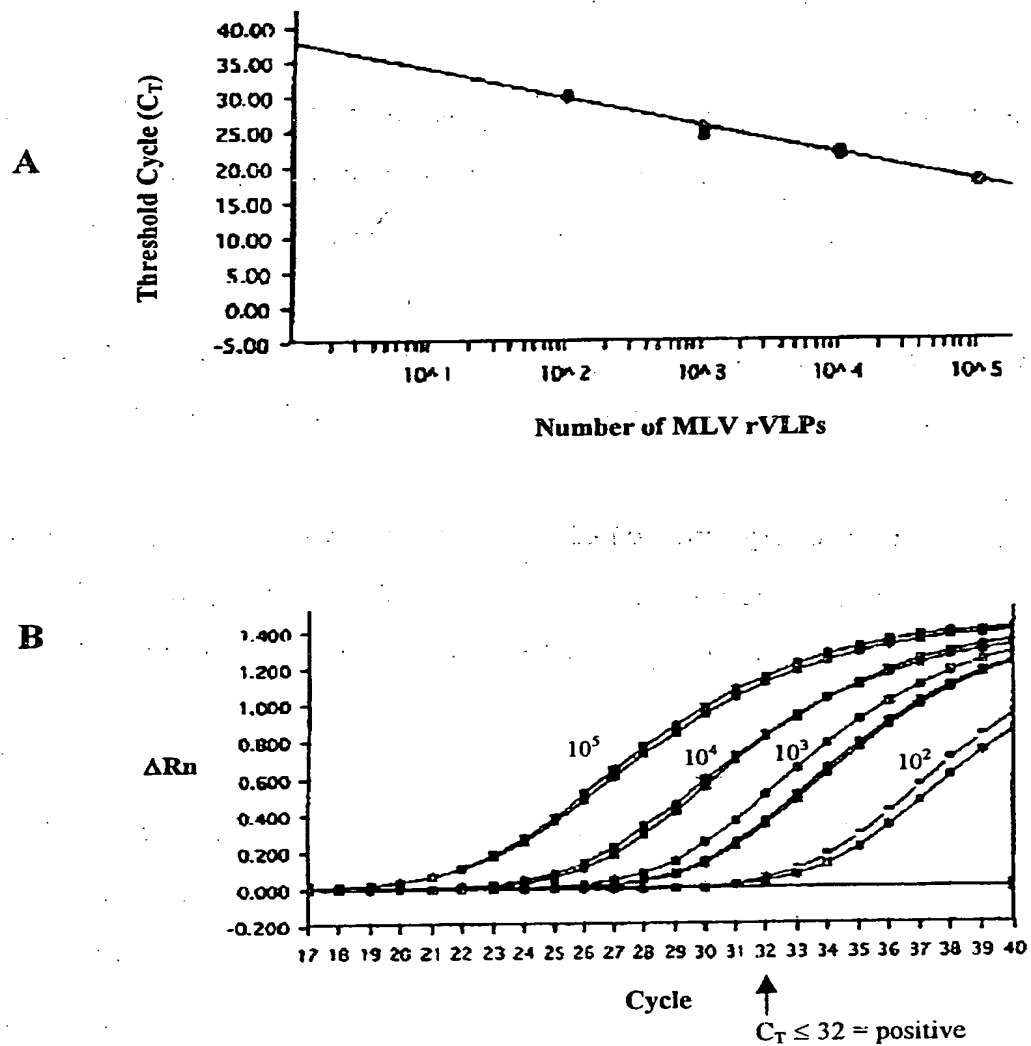
35. A kit according to claim 34 wherein the oligonucleotides are PoERV-gag-specific oligonucleotides derived from sequences which correspond to approximate positions 805-823 (forward), to positions 828-854 (probe) and positions 902-877 (reverse) on the PoERV genome.

36. A kit according to claim 35 wherein the oligonucleotide primers for the amplification are:

Forward primer 5'-CCGGCTCTCATCCTGATCA-3'

Reverse primer 5'-TCTTGTTTATTTAGCCATGGTTTAA-3'.

FIGURE 1



2/7

FIGURE 2

Cell Line / Virus	Description	Identification / Source	
BHK-21	Baby hamster kidney	CCL 10	ATCC
Vero	African green monkey	CRL 1586	ATCC
MRC-5	Human fibroblast	CCL 171	ATCC
Raji	Human lymphocyte	CCL 86	ATCC
BT	Bovine turbinate	CRL 1390	ATCC
<i>Mus dunni</i>	Normal mouse tail fibroblast	CRL 2017	ATCC
K-Balb	Mouse embryo sarcoma	89092101	ECACC
Sf9	<i>Spodoptera frugiperda</i> ovary	Cat # B825-01	Invitrogen
NSO	Mouse lymphoblast myeloma	85110503	ECACC
CHO-K1	Chinese hamster ovary	CCL 61	ATCC
SP2/0-Ag14	Mouse lymphoblast myeloma	85072401	ECACC
C127I	Mouse mammary	90060504	ECACC
PoERV	<i>Porcine endogenous retrovirus</i>	pK15(CCL 33)	ATCC
MLV	<i>Murine leukaemia virus</i>	Strain Mov-3	DVPGU.
SMRV	<i>Squirrel monkey retrovirus</i>	VR 843	ATCC
SIV _{mac}	<i>Simian immunodeficiency virus</i>	Cat #10149001	ABInc
SRV-D	<i>Simian type-D retrovirus 1</i>	Cat # 10152001	ABInc
SFV	<i>Simian foamy virus</i>	VR 276	ATCC

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3/7

FIGURE 3

A

Sample	PoERV rVLPs	MLV rVLPs	SMRV rVLPs
No RT	-/- (37-40 ^a) (39-40)	-/- (40-40 ^b) (40-40)	-/- (40-40 ^c) (39-40)
10 ⁴ rVLP	+/+(30-33 ^a) (25-31)	+/+(27-28 ^b) (23-24)	+/+ (21-23 ^c) (23-24)
10 ³ rVLP	-/+ (35-35 ^a) (29-30)	+/+ (31-32 ^b) (26-28)	+/+ (25-26 ^c) (28-28)
10 ² rVLP	-/+ (36-40 ^a) (29-33)	-/+ (34-35 ^b) (30-32)	+/+ (29-30 ^c) (29-30)
10 rVLP	-/- (37-40 ^a) (33-34)	-/- (39-40 ^b) (35-37)	+/+ (32-33 ^c) (32-34)

B

Sample	MLV RT	AMV RT
No RT	-(36-40)	- (36-40)
10 ³ molecules	+(23-25)	+ (26-28)
10 ² molecules	+(26-27)	+ (27-29)
10 molecules	+(27-28)	+(27-33)

FIGURE 4

Retrovirus	Cation Preference	Detection Assay	
		RT	F-PERT
MLV	Mn ²⁺	Neat	10 ⁻⁵
PoERV	Mn ²⁺	10 ⁻¹	10 ⁻⁵
SFV	Mn ²⁺	Neat	10 ⁻⁶
SIV _{mac}	Mg ²⁺	10 ⁻¹	10 ⁻⁶
SMRV	Mg ²⁺	Neat	10 ⁻⁵
SRV-1 type D	Mg ²⁺	Neat	10 ⁻⁵

FIGURE 5

Divalent Cation	Concentration(mM)	10 ⁶ MLV rVLPs		10 ⁶ PoERV rVLPs	
		Mean	CL	Mean	CL
Mn ²⁺	0.15	17.1	(15.5-18.7)	22	(19.0-25.0)
	0.5	17.4	(12.9-21.8)	20.6	(11.6-29.7)
	1.0	17.3	(14.6-20.1)	18.5	(15.9-21.0)
	1.5	17.4	(13.5-21.3)	17.6	(14.2-20.9)
	2.0	16.4	(11.6-21.1)	16.7	(15.7-17.5)
Mg ²⁺	5.0	16.8	(16.5-17.1)	16.3	(16.2-16.5)
	7.5	NT	NT	19.5	(19.4-19.6)
	10.0	NT	NT	19.1	(19.0-19.2)

FIGURE 6

A

1000ng MRC-5 Total Cell Lysate						
BMV : aCT DNA BMV (ng)	1:26 60	1:52 30	1:104 15	1:209 7	1:418 3.5	1:836 1
+ aCT DNA	+	+	-	-	-	-
- aCT DNA	+	+	-	-	-	-
Spiked 10 ³ VLP	+	+	+	+	-	-
10 ³ VLP+ aCT	+	+	+	+	+	+
10 ³ VLP- aCT	+	+	+	+	+	+

B

Sample	α	β	δ	Taq	TdT	SMRV
Minus aCT	37-40	40-40	26-28	22-31	40-40	22-29
Plus aCT	40-40	40-40	39-40	40-40	40-0	26-28

7/7

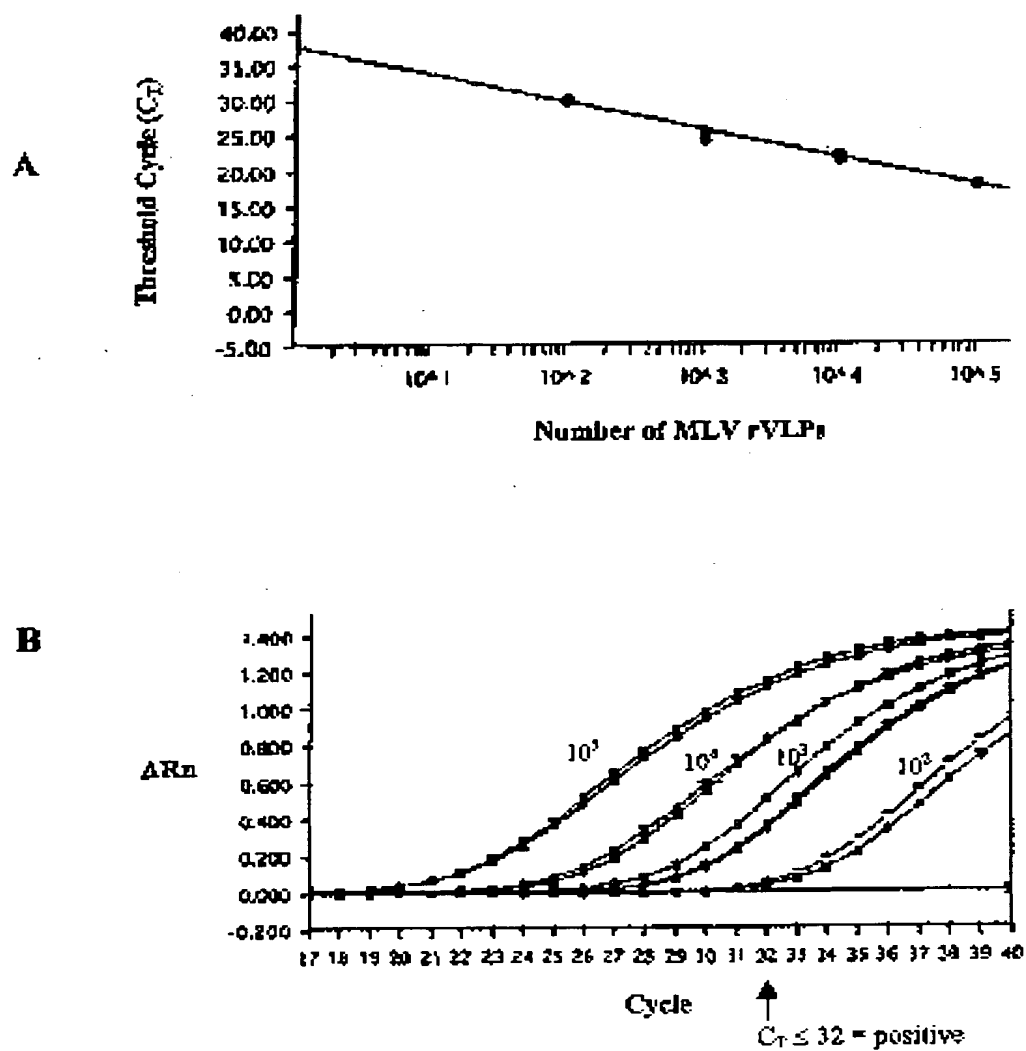
FIGURE 7

Sample	Sentinel	With aCT DNA	Without aCT DNA	Spiked sample
Vero	38-40 (-)	35-40 (-)	27-31 (+)	21-28 (+)
Raji	39-40 (-)	36-40 (-)	36-37 (-)	27-27 (+)
MRC-5	40-40 (-)	NT	40-40 (-)	15-18 (+ ^b)
BT	40-40 (-)	NT	37-41 (-)	16-18 (+ ^b)
<i>Mus dunni</i>	40-40 (-) 40-40 (- ^a) IDU	33-36 (-) 27-31 (+ ^a) IDU	29-32 (+) 21-24 (+ ^a) IDU	29-32 (+ ^a) 29-32 (+ ^a)
C127I	40-40 (-) 40-40 (- ^a) IDU	35-36 (-) 29-30 (+ ^a) IDU	33-38 (-) 24-25 (+ ^a) IDU	28-29 (+) 29-32 (+ ^a) IDU
Sf-9	39-40 (-)	26-27 (+)	23-24 (+)	26-27 (+)
BalbC	40-40 (-) 40-40 (- ^a) IDU	30-33 (+) 20-22 (+ ^a) IDU	31-34 (+) 17-19 (+ ^a) IDU	20-33 (+) 30-32 (+ ^a) IDU
NS0	39-40 (-)	20-27 (+)	NT	NT
Sp2/0-Ag14	39-40 (-)	19-23 (+)	NT	NT
BHK-21	39-40 (-)	24-25 (+)	20-21 (+)	26-26 (+)

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1/7

FIGURE 1



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2/7

FIGURE 2

Cell Line / Virus	Description	Identification / Source	
BHK-21	Baby hamster kidney	CCL 10	ATCC
Vero	African green monkey	CRL 1586	ATCC
MRC-5	Human fibroblast	CCL 171	ATCC
Raji	Human lymphocyte	CCL 86	ATCC
BT	Bovine turbinate	CRL 1390	ATCC
<i>Mus dunni</i>	Normal mouse tail fibroblast	CRL 2017	ATCC
K-Balb	Mouse embryo sarcoma	69092101	ECACC
Sf9	<i>Spodoptera frugiperda</i> ovary	Cat # B825-01	Invitrogen
NSO	Mouse lymphoblast myeloma	85110503	ECACC
CHO-K1	Chinese hamster ovary	CCL 61	ATCC
SP2/C-Ag14	Mouse lymphoblast myeloma	85072401	ECACC
C127	Mouse mammary	90060504	ECACC
PoERV	<i>Porcine endogenous retrovirus</i>	pK15(CCL 33)	ATCC
MLV	<i>Murine leukaemia virus</i>	Strain MoMu-3	DVPGU
SMRV	<i>Squirrel monkey retrovirus</i>	VR 843	ATCC
SIV _{mac}	<i>Simian immunodeficiency virus</i>	Cat #10149001	ABIno
SRV-D	<i>Simian type-D retrovirus 1</i>	Cat # 10152001	ABIno
SPV	<i>Simian foamy virus</i>	VR 276	ATCC

SUBSTITUTE SHEET (RULE 26)

3/7

FIGURE 3

A

Sample	PoERV rVLPs	MLV rVLPs	SMRV rVLPs
No RT	-/- (37-40 ^a) (39-40)	-/- (40-40 ^b) (40-40)	-/- (40-40 ^a) (39-40)
10 ⁴ rVLP	+/+ (30-33 ^a) (25-31)	+/+ (27-28 ^b) (23-24)	+/+ (21-23 ^c) (23-24)
10 ³ rVLP	-/+ (35-35 ^a) (29-30)	+/+ (31-32 ^b) (26-28)	+/+ (25-26 ^c) (28-28)
10 ² rVLP	+/+ (36-40 ^a) (29-33)	+/+ (34-35 ^b) (30-32)	+/+ (29-30 ^c) (29-30)
10 rVLP	-/- (37-40 ^a) (33-34)	-/- (39-40 ^b) (35-37)	+/+ (32-33 ^c) (32-34)

B

Sample	MLV RT	AMV RT
No RT	-(36-40)	-(36-40)
10 ³ molecules	+(23-25)	+(26-28)
10 ² molecules	+(26-27)	+(27-29)
10 molecules	+(27-28)	+(27-33)

FIGURE 4

Retrovirus	Cation Preference	Detection Assay	
		RT	F-PERT
MLV	Mn ²⁺	Neat	10 ⁻³
PoERV	Mn ²⁺	10 ⁻¹	10 ⁻³
SPV	Mn ²⁺	Neat	10 ⁻⁶
SIV _{mac}	Mg ²⁺	10 ⁻¹	10 ⁻⁶
SMRV	Mg ²⁺	Neat	10 ⁻³
SRV-1 type D	Mg ²⁺	Neat	10 ⁻⁵

FIGURE 5

Divalent Cation	Concentration(mM)	10 ² MLV rVLPs		10 ³ PoERV rVLPs	
		Mean	CL	Mean	CL
Mn ²⁺	0.15	17.1	(15.5-18.7)	22	(19.0-25.0)
	0.5	17.4	(12.9-21.8)	20.6	(11.6-29.7)
	1.0	17.3	(14.6-20.1)	18.5	(15.9-21.0)
	1.5	17.4	(13.5-21.3)	17.6	(14.2-20.9)
	2.0	16.4	(11.6-21.1)	16.7	(15.7-17.5)
Mg ²⁺	5.0	16.8	(16.5-17.1)	16.3	(16.2-16.5)
	7.5	NT	NT	19.5	(19.4-19.6)
	10.0	NT	NT	19.1	(19.0-19.2)

FIGURE 6

A

1000ng MRC-5 Total Cell Lysate						
BMV + α CT DNA	1:24	1:52	1:104	1:209	1:418	1:836
BMV (ng)	60	30	15	7	3.5	1
+ α CT DNA	+	+	-	-	-	-
- α CT DNA	+	+	-	-	-	-
Spoken 10^3 VLP	+	+	+	+	-	-
10^3 VLP + α CT	+	+	+	+	+	+
10^3 VLP - α CT	+	+	+	+	+	+

B

Sample	α	β	δ	Taq	TdT	SMRV
Minus α CT	37-40	40-40	26-28	22-31	40-40	22-29
Plus α CT	40-40	40-40	39-40	40-40	40-0	26-28

SUBSTITUTE SHEET (RULE 26)

7/7

FIGURE 7

Sample	Sentinel	With aCT DNA	Without aCT DNA	Spiked sample
Vero	38-40 (-)	35-40 (-)	27-31 (+)	21-23 (+)
Raji	39-40 (-)	36-40 (-)	36-37 (-)	27-27 (+)
MRC-5	40-40 (-)	NT	40-40 (-)	15-18 (+ ^b)
BT	40-40 (-)	NT	37-41 (-)	16-18 (+ ^b)
<i>Mus dunni</i>	40-40 (-) 40-40 (- ^b) IDU	33-36 (-) 27-31 (+ ^a) IDU	29-32 (+) 21-24 (+ ^b) IDU	29-32 (+) 29-32 (+ ^b)
C1271	40-40 (-) 40-40 (- ^b) IDU	35-36 (-) 29-30 (+ ^b) IDU	33-38 (-) 24-25 (+ ^b) IDU	28-29 (+) 29-32 (+ ^b) IDU
Sf-9	39-40 (-)	26-27 (+)	23-24 (+)	26-27 (+)
BalbC	40-40 (-) 40-40 (- ^b) IDU	30-33 (+) 20-22 (+ ^a) IDU	31-34 (+) 17-19 (+ ^b) IDU	20-33 (+) 30-32 (+ ^b) IDU
NSO	39-40 (-)	20-27 (+)	NT	NT
Sp2/O-Ag14	39-40 (-)	19-23 (+)	NT	NT
BHK-21	39-40 (-)	24-25 (+)	20-21 (+)	26-26 (+)

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